

Automation of a flocculation test for syphilis on Groupamatic Equipment

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The widespread utilization of Groupamatic Equipment for routine immunohaematological tests has increased the demand for serological procedures that can be performed with this machine. We now use two Groupamatic 360 GC machines on which we process each year more than 250,000 blood samples (Garretta, Gener, Muller, Matte, and Moullec, 1974a). These considerations make it apparent that automation of routine syphilis tests should be investigated.

In the serology of syphilis, the continuous-flow auto-analyser system has been applied to complement-fixation techniques. In 1968, the Venereal Disease Research Laboratory, in cooperation with Technicon Corporation, developed an automated flocculation test for syphilis (McGrew, Ducros, Stout, and Falcone, 1968a; Stout, McGrew, and Falcone, 1968). Originally designed for a single-channel continuous-flow system, it has been adapted to a multichannel system (Schroeter, Taswell, Kierland, and Sweatt, 1971). In both, the sensitivity and specificity appeared to be greater than in the manual VDRL test (McGrew, Stout, and Falcone, 1968b; Stevens and Stroebel, 1970). Until the beginning of 1974, the routine screening at the Centre National de Transfusion Sanguine (CNTS) was effected on two single-channel continuous-flow systems using a cardiolipid antigen coated on inert particles‡. The reactive samples were checked by a modified rapid plasma reagin (RPR) slide test (read microscopically), and then sent for verification to the Alfred Fournier Institute. Under these conditions, we found in 1973 a positive rate of 0.8‰ and a false positive rate of 2‰.

This paper describes the test method which was developed at the CNTS. We use a lipoidal antigen

made up at the CNTS from the commercial K-antigen of Lille. Preliminary investigations (Garretta, Paris-Hamelin, Gener, Muller, Matte, Vaisman, and Moullec, 1973b) showed that this technique could be applied to a serological test for syphilis and we have been able to use it routinely since January, 1974, for all blood donors. To evaluate the new automated procedure, the test was performed on more than 63,000 samples. For 5,212 of them, the results were compared with those of classical serology and with indirect immunofluorescence methods (FTA-200 and FTA-ABS) and with the Nelson and Mayer test (TPI). All the positive results were confirmed, but in the Tables only the results concerning classical serological screening methods are shown.

Material and methods

EQUIPMENT

We use two Groupamatic 360 GC§ machines the construction of which is now well known (Garretta, Muller, Gener, Matte, and Moullec, 1974b). Fig. 1 shows the basic layout of this equipment. It enables 340 blood samples to be analysed per hour in twelve separate channels including an electromechanical unit and an electronic unit. The former is used for agglutination reactions and identification of the samples. The latter, with a software programme, processes the data and prints them out. We use up to five successive runs, the 4th and 5th only concerning our most regular donors. The first and second runs are made in programme P₁ and the tests performed are ABO group, with determination of A₁, A₂, B, O, A₁B and A₂B; Rh factor with determination of D and D^u; detection of rh' and rh''; detection of irregular alloantibodies; detection of immune antibodies anti-A and anti-B; screening test for syphilis. The third run uses the P₂ programme for identification of detected alloantibodies. The fourth and fifth runs are used with programme P₃ for erythrocyte phenotypings: K, C, c, D, D^u, E, e, C^w, Le (a), P₁, M and N.

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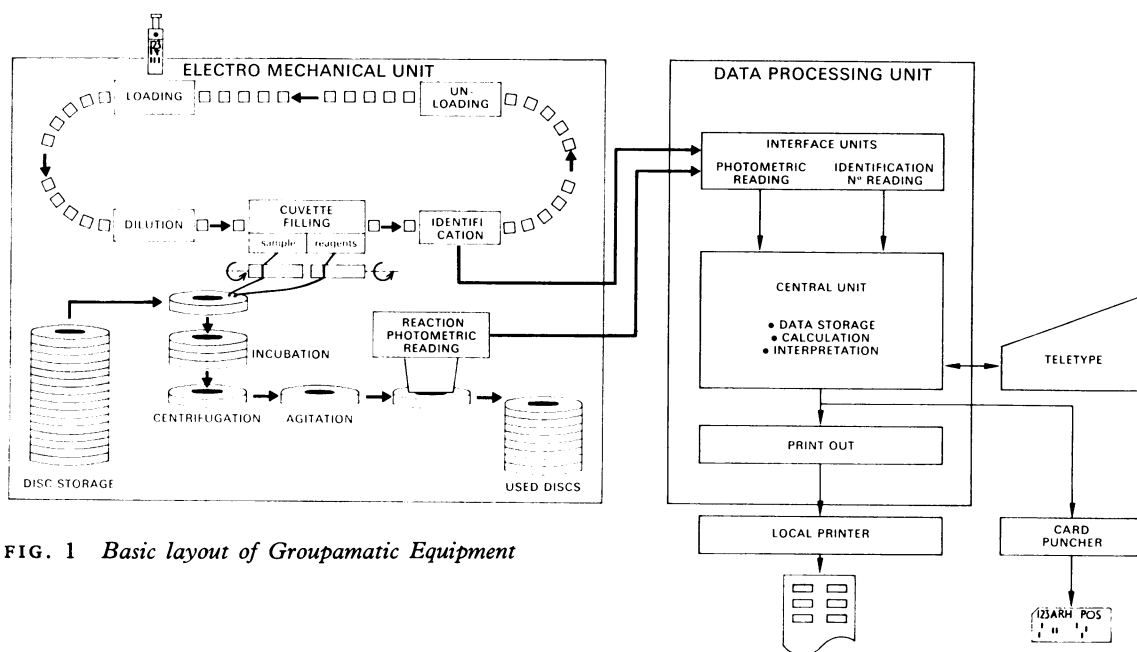


FIG. 1 Basic layout of Groupamatic Equipment

One important point is the photometric reading of the reactions. The liquid mass contained within each cuvette is submitted to two measurements of opacity: a central reading and a peripheral annular reading. Each central measurement is classed as positive or negative by comparison with a threshold level. Each peripheral measurement is classed as negative, positive, or weakly positive by comparison with two threshold levels, a low and a high. The central and peripheral measurements are compared, and a negative response is characterized by absence of any variation in light intensity. On the contrary, a normal positive response includes some variation in opposite directions.

The detection of syphilis is made in programme P₁ in channel 10. The grouping data give rise to a black printing of digits from 0 to 9, which are a function of the absence or presence of agglutination. The results are now printed out on self-adhesive labels and simultaneously on an 80-column punched card through the IBM card punch machine. The central threshold is adjusted at 3, the low peripheral threshold at 2, and the high peripheral threshold at 3. The central calibration is identical with that used for haemagglutination. The peripheral calibration is made in two steps on a real test: on a negative reaction for the peripheral zero, and on a one plus positive reaction for the peripheral maximum. Lastly, simultaneously with the acquisition and print-out of the characteristics of the reactions, an electromechanical reader records the identification number of the corresponding sample. The results are, in this way, attributed to the tested sample with no possibility of error.

REAGENT

We use a reagent developed in the CNTS laboratories, the GAST* from the K antigen prepared by M. G.

Chateau at the Regional Blood Transfusion Centre in Lille. Antigen for this test is an alcoholic solution containing cardiolipin, cholesterol, and colloidal benzoin gum. It is coloured by amido black and this stabilized suspension is calibrated to about 2 μ . The antigen-coated inert particles are placed in a special medium permitting collection of possible agglutinates. This reagent is now produced commercially in Lille and each lot of antigen is serologically standardized by proper comparison with an antigen of known reactivity. It is kept at + 4°C., but must be used at room temperature (+ 18° to + 23°C.), either by rewarming it in a water-bath for 20 min. at + 37°C., or by leaving it at least 4 hrs at laboratory temperature. The reaction works in one step; one aliquot of 0.1 ml. of plasma is mixed in the cuvette with one aliquot of 0.1 ml. of GAST.

SAMPLES TESTED

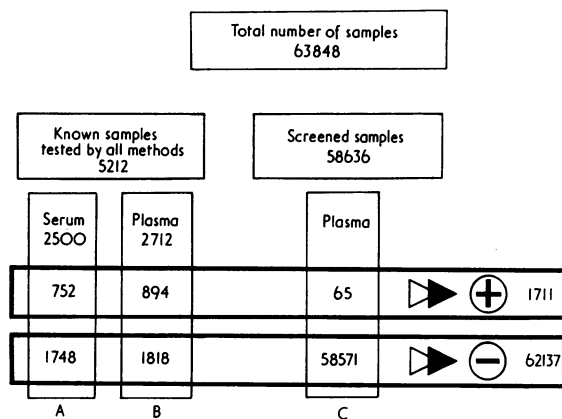
63,848 specimens were classified into two categories: known samples already tested by all methods and screened samples (Fig. 2).

The known samples came from the Alfred Fournier Institute and had been stored frozen at - 20°C. at the CNTS. They were accidentally frozen and thawed and we were compelled to use as a reference the modified RPR slide test. The first part (A) contained 2,500 samples of sera, 752 of which were positive; the second part (B) comprised 2,712 samples of plasma, 894 of which were positive.

The 58,636 screened samples were all obtained from volunteer blood donors (C). As in the case of the other plasma samples, they were collected in Vacutainers†

*Groupamatic automated syphilis test

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FIG. 2 *Specimens tested during study*

containing potassium EDTA (0.06 ml. of a 15 per cent. solution in a 7 ml. tube); this collection system has the double advantage of a strictly constant level and almost total absence of small blood clots. Specimens of Series A and B were identified with serial code numbers. Specimens of Series C were identified with the Groupamatic identification number, given before collection. The syphilis group included samples from both treated and untreated patients.

TEST PROCEDURES

All the tests were performed independently either at the Alfred Fournier Institute or at the CNTS, the results being compared later. Specimens of Series A and B were tested on serum at the Alfred Fournier Institute by the following reactions: Kolmer CF, Reiter CF (RPCF), Kahn, Kline. The positive results were confirmed by the TPI test, by the FTA-200 and FTA-ABS tests, and, in some cases, by a haemagglutination reaction (TPHA). All these tests were performed according to published techniques. At the CNTS a modified RPR slide test was used as a reference (Portnoy, Brewer, and Harris, 1963). For completeness in the evaluation, we also performed an automated flocculation test on the auto-analyser continuous-flow system (single-channel) and these results

will be briefly discussed at the end of this report. All the specimens of Series C were also tested on single-channel continuous flow systems, with checking of positives by the RPR test. Here, also, the positive results were confirmed by fluorescent antibody and treponemal tests.

Results

After preparation, the reactions may appear as follows (Fig. 3):

(1) Normal negative reaction: slightly bluish turbid and homogeneous liquid mass.

(2) Doubtful positive reaction \pm : a little central opaque spot surrounded by a first peripheral area relatively opaque and a second turbid peripheral area.

(3) Positive reaction $+$: central spot of about 4 mm., very opaque, surrounded by a less transparent area.

(4) Positive reaction $++$: central spot of about 8 mm., very opaque, surrounded by a very transparent area.

SERIES A. KNOWN SERUM SAMPLES TESTED BY ALL METHODS (Table I)

(1) 555 positive specimens were at least $++$ in modified RPR slide test

The agreement of the GAST and RPR tests on the 555 specimens in this study was 99.8 per cent. Only one positive sample was not detected, but it has not been possible to test it again.

(2) 134 positive samples were $+$ to \pm in modified RPR slide test

Seven specimens (5.2 per cent.) were not detected, mainly corresponding to the weaker doubtful reactions in the modified RPR test.

(3) 63 positive samples were $+$ to \pm in modified RPR slide test, but were sometimes negative in traditional screening tests

The percentage of false negatives was 4.8 per cent. (three samples out of 63). These three specimens also gave weak doubtful reactions in the modified RPR test.

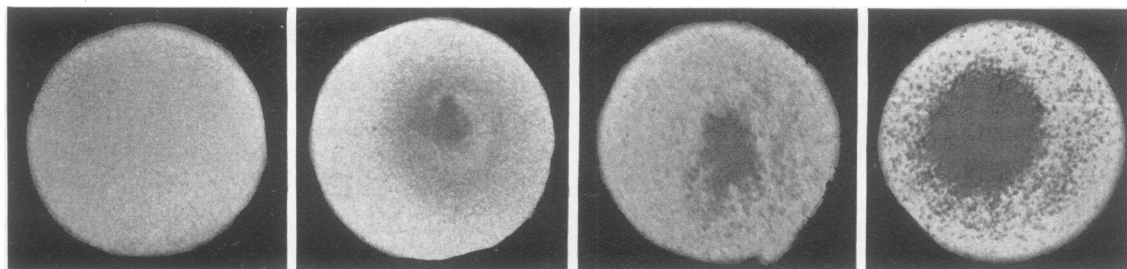


FIG. 3 *Contents of cuvettes after agitation phase. From left to right: normal negative reaction; doubtful positive reaction (\pm); positive reaction ($+$); positive reaction ($++$)*

TABLE I *Series A Tests performed on serum samples*

| Number of samples | Tests performed on fresh serum | | | | Tests performed on serum after freezing-thawing (once) | | |
|-------------------------------|--------------------------------|----------------|---------------|---------------|--|-----------------------|----------------------|
| | Reiter | Kolmer | Kline | Kahn | Modified RPR (Manual) reference test | Groupamatic | |
| | | | | | | + | — |
| 555 | NT | +++ | +++ | +++ | ++ | 554 | 1 (0.2 per cent.) |
| 134 (Doubtful reactions) | NT | +++ to + | + to ± | + to ± | + to ± | 127 | 7 (5.2 per cent.) |
| 63 (Dissociated reactions) | NT | ++ to — | ++ to — | ++ to — | + to ± | 60 | 3 (4.8 per cent.) |
| 1,748 | NT | — | — | — | NT | 45 (2.6 per cent.) | 1,703 |

NT = Not tested

(4) 1,748 negative specimens

45 samples (2.6 per cent.) were found to be falsely positive. Some of them were only spotted by visual control, the automatic reading being negative.

SERIES B. KNOWN PLASMA SAMPLES TESTED BY ALL METHODS (Table II)

It was in this series that the samples had been accidentally frozen and thawed, and the difference

in results between the tests performed on fresh serum at the Alfred Fournier Institute and those performed on plasma at the CNTS were sometimes fairly large. This is why we used the modified RPR slide test as a reference test.

(1) 389 positive samples were $\geq +$ in modified RPR slide test

384 specimens out of 389 were detected; the per-

TABLE II *Series B Tests performed on plasma samples*

| Number of samples | Tests performed on fresh serum | | | | Tests performed on plasma after freezing-thawing (several times) | | |
|--------------------------------|--------------------------------|-----------------|-----------------|-----------------|--|-----------------------|-------------------------|
| | Reiter | Kolmer | Kline | Kahn | Modified RPR (Manual) reference test | Groupamatic | |
| | | | | | | + | — |
| 389 | +++ to ++ | +++ to ++ | +++ to ++ | +++ to ++ | +++ to + | 384 | 5 (1.3 per cent.) |
| 232 (Doubtful reactions) | +++ to ++ | +++ to + | +++ to ± | +++ to ± | ± — 134 98 (42.2 per cent.) | 71 | 161 (69.4 per cent.) |
| 30 (Dissociated reactions) | +++ to — | +++ to — | +++ to — | +++ to — | ++ to + | 25 | 5 (16.7 per cent.) |
| 228 (Dissociated reactions) | +++ to — | +++ to — | ++ to — | ++ to — | + — 111 117 (51.3 per cent.) | 49 | 179 (78.5 per cent.) |
| 15 (Doubtful reactions) | AC | AC | +++ to ± | +++ to ± | + — 11 4 (26.7 per cent.) | 11 | 4 (26.7 per cent.) |
| 1,615 | — | — | — | — | NT | 72 (4.5 per cent.) | 1,543 |
| 69 | AC | AC | — | — | NT | 2 (2.9 per cent.) | 67 |
| 194 | +++ to + | — | — | — | NT | 6 (3.1 per cent.) | 188 |

NT = Not tested

AC = Anticomplementary

centage of false negatives was 1.3. Here again, it was impossible to re-test these samples.

(2) 232 positive samples were \pm to $-$ in modified RPR slide test

We noticed that the majority of these samples gave doubtful Kline and Kahn reactions on fresh serum. Out of 232 positive samples, only the 134 (57.8 per cent.) RPR positive samples can be considered as positive for the purposes of our comparison. Only 71 were detected with the GAST, which represents 30.6 per cent. of the total number, or 52.9 per cent. of the number of RPR positive samples.

(3) 30 positive samples were $\geq +$ in modified RPR slide test but were negative in one or several traditional tests

The percentage of false negatives was 16.7 per cent. (five samples out of thirty); all gave negative reactions in both Kline and Kahn tests.

(4) 228 positive samples were \pm to $-$ in modified RPR slide test, but were negative in one or several traditional tests

Only the 111 samples detected with the modified RPR can be considered as positive (48.7 per cent. of all the specimens). We detected only 49 samples with the GAST, i.e. 44.1 per cent. (21.5 per cent. of all the specimens).

(5) 15 positive samples, all of which were anti-complementary in haemolysis reactions on fresh serum
Eleven out of fifteen were detected (73.3 per cent.)
The four false negative samples all gave doubtful reactions in traditional flocculation tests.

(6) 1,615 negative specimens

72 samples were considered as falsely positive (4.5 per cent.). More than half of them were spotted only by visual control and not by the machine.

(7) 69 negative specimens, all of which were anti-complementary in haemolysis reactions on fresh serum
The percentage of false positives was 2.9 per cent. (two samples out of 69).

(8) 194 negative specimens, but all giving false positive results in haemolysis reactions on fresh serum

Six samples out of 194 were detected, i.e. 3.1 per cent. of false positives with the GAST.

SERIES C. SCREENED SAMPLES (Fig. 4)

58,636 samples were tested. 65 (1.1 per 1,000) were detected and found positive in the FTA and TPI. Only one of them was $< +$ on the Groupamatic machine, the 64 others being $\geq +$. We checked 2,852 samples which were not completely negative on the Groupamatic, but were detected by the automatic reading or visually. All these samples were negative with the modified RPR manual test, as well as the TPI and the FTA. Among these, only 856 were considered to be true false positives, i.e. 1.4 per cent.

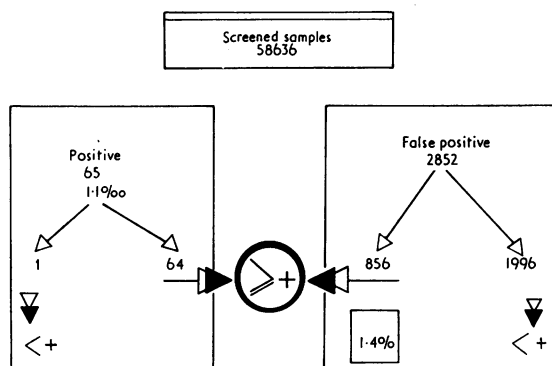


FIG. 4 Series C—Results of screened samples

of all the plasmas tested. The others were all $< +$ on the machine and most of these readings were caused by electromechanical unit problems, in particular stopping of the conveyor belt. This point is very important for the automatic reading of the reaction: there is no problem at all for the photometer to detect a reaction $\geq +$ with the GAST. We noticed that all the positives were in fact $\geq +$.

REPRODUCIBILITY OF THE GAST

Evidence for the reproducibility of the GAST is presented in Fig. 5. In these experiments, forty selected specimens were tested each time we processed the GAST. Except for one doubtful result, complete agreement was obtained in all cases.

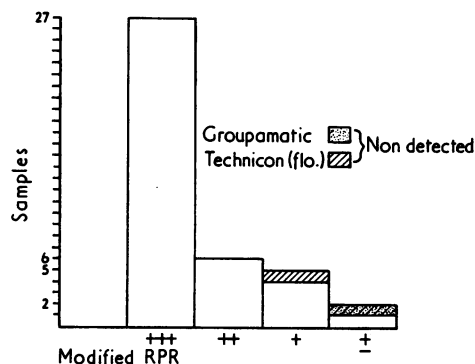


FIG. 5 Reproducibility of GAST on forty selected specimens tested repeatedly

RELATION BETWEEN THE GROUPAMATIC AUTOMATIC READING AND THE VISUAL INTERPRETATION (Fig. 6)
We selected a sample giving $+++$ with the modified RPR slide test and performed nine dilutions from 1/10 to 9/10. There is a close relationship between the two kinds of readings. There is no problem for the Groupamatic machine except for the doubtful

the percentage of Groupamatic false negatives was 0.18 per cent., which represents actually only one discrepancy in a sample which could not be tested again. Thus the results are excellent for this set of samples. As far as weak and doubtful reactions are concerned, the percentages of false negative samples with the Groupamatic were respectively 5.22 and 4.76 per cent. which is quite reasonable for an automatic screening test.

As far as Series B is concerned, all samples tested at the CNTS were of plasma collected with EDTA-K3. They underwent several freezing/thawing processes and clear discrepancies appear between the RPR performed on these plasma samples and flocculation reactions initially performed on fresh sera. Detection of weak, moderate, and strong positive samples on the Groupamatic did not raise any problem: the percentage of false positives was estimated at 1.3. As far as very weakly positive or doubtful samples are concerned, we find the same problems as before, the percentages of false negatives being respectively 47, 16.7, 55.8, and 0 per cent. (see Table II). The significance of these samples is a matter of opinion in relation to transfusion, but one should take into consideration the inferior sensitivity of the GAST reagent for samples giving dissociated or doubtful reactions with the four usual screening techniques.

The percentage of false positives found in Series A was 2.6 per cent. and in the three groups of Series B 4.5, 2.9, and 3.1 per cent. respectively. It should be mentioned that we have considered as false positive all reactions which were not strictly and homogeneously negative, which explains this relatively high number of false positives. On the other hand, we have been able to make a detailed study of 2,852 false positive results found among 58,636 samples tested in Series C. Only 856 of them (1.4 per cent.) were true false positives. This is very reasonable for a screening procedure, since these samples are tested again by manual techniques.

It seems to us that this automated screening technique on Groupamatic systems, using a cardiolipid antigen, meets the needs of a Blood Transfusion Centre. Sensitivity is very good for strong reactions (more than +) but low for samples with doubtful or dissociated reactions. Reproducibility among batches was tested on forty samples of different reactivity and appeared to be excellent (see Fig. 5). Automatic reading of reactions is fair. The relations, for different dilutions of a positive plasma, between the reactivity examined by the charcoal RPR technique read under the microscope and the automatic reading by the Groupamatic are given in Fig. 6. No problem appeared except for a doubtful sample, the reading of which was below the threshold of the photometer. This confirms that a quick visual check of every disc is worthwhile, as is done by us for

immunohaematological reactions. It would be possible to detect these samples automatically, by decreasing the low threshold of the photometer, but this would not be acceptable, since micro-strings of fibrin appear even when collection is by Vacutainer. Prints-out up to 2 can then appear, which is not compatible with routine work (Garretta, Muller, André, Moullec, and Matte, 1973b).

All of these results demonstrate the very clear advantages of the Groupamatic over the continuous-flow techniques. They bear most of all on the absence of contamination between samples, the automatic reading and printing-out of results, and the rapidity of the reactions in that 340 tests are performed per hr. In our preliminary report (Garretta and others, 1973a), we compared the results of the mono-channel Technicon and the Groupamatic, and found for the former a percentage of false positives of 3 to 4 per cent. The GAST sensitivity for strongly positive samples was already excellent, which was not always the case with our single-channel autoanalyzers, as the size of the clump did not allow it to pass through the appropriate tubing; this problem had already been raised by other workers, notably by Schroeter and others (1971). Finally, we had the same difficulties with the weak samples no matter what the technique. When the final results for the autoanalyzers were reviewed, they proved to be much worse than we had imagined in certain series, especially as regards the false negatives. In Series A, the number of false negatives was 18, 62, and 65 per cent.; in Series B, this figure varied between 17 and 63 per cent. depending on the sample groups. The number of false positives in Series A was 3.4 per cent.; and that in Series B varied between 8.6 and 12.3 per cent.

The results obtained with our autoanalyzers, under conditions quite similar to those of the GAST evaluation, and assessed blindly, were much inferior. These results relate to 5,212 samples of which 1,646 were positive. What explanation is there? The first possibility concerns the way the machines are used; it appears, however, that their use is sufficiently simple for our staff so that their operation should be error-free. Again, the explanation does not seem to lie with the antigen; the GAST includes, as basic antigen, the same antigen used in the continuous-flow autoanalyzer, but with much better results. Finally, the tested samples were identical with the two automatic techniques. We can but state these results and bring to mind what some of us have found recently (Paris-Hamelin, Vaisman, and Fustec-Ibarboure, 1974) regarding the basic antigen used in the RPR card test and the ART (automatic reagin test); this antigen used manually on plasma, or on serum gave excellent results; the use of a continuous-flow autoanalyzer was so disappointing that it had to be given up. The ART antigen was not at fault, but rather the

modified technique for this type of automatic equipment.

Bessemans and Derom (1946) showed that blood, serum, and plasma were capable, even in small amounts, of transmitting syphilis. They insisted on the now obvious fact that apparent health and negative serology did not guarantee the safety of the blood. Mollison (1972) stated that 35 per cent. of cases of primary syphilis are serologically negative. One of us (Vaisman, 1958) estimated that the phases during which blood is most infectious are the incubation phase, the primary phase, and the secondary phase. There is therefore no serological safety net during the incubation phase and for part of the primary stage. The data concerning the survival time of *T. pallidum* under conditions of blood transfusion are contradictory, and we are now working on this subject. Furthermore, an investigation involving dermatologists, neurologists, and cardiologists is planned, in which recipients who have been exposed to risk of infection will be followed-up.

Summary

A flocculation reaction employing a cardiolipid antigen was used for syphilis screening on Groupamatic equipment in parallel with conventional screening reactions: Kolmer CF, RPCF, Kahn, Kline, and RPR. The positive samples were confirmed by FTA-200, FTA-ABS, TPI, and in some cases by TPHA.

There were 5,212 known samples which had already been tested by all methods and of which 1,648 were positive, and 58,636 screened samples including 65 positives. Half of the samples in the first series were taken without anticoagulant; the remainder were collected in potassium EDTA.

The percentage of false positives with the Groupamatic was about 1.4 per cent. The percentage of false negatives among positive ($\geq +$) samples varied from 0.18 to 1.3 per cent.; on the other hand the sensitivity was less good for samples giving doubtful and/or dissociated reactions in conventional screening reactions.

The specificity and sensitivity of this technique are acceptable for a blood transfusion centre. The reproducibility is excellent and the automatic reading of results accurate. Additional advantages are rapidity (340 samples processed per hour); simultaneous performance of eleven other immunohaematological reactions; no contamination between samples; automatic reading, interpretation, and print-out of results; and saving of time because samples are not filed sequentially and are automatically identified when the results are obtained.

Although the importance of syphilis in blood transfusion seems small, estimates of the risk are difficult and further investigations are planned.

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